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Do environmental factors affect male fathead minnow (*Pimephales promelas*) response to estrone? Part 2. Temperature and food availability

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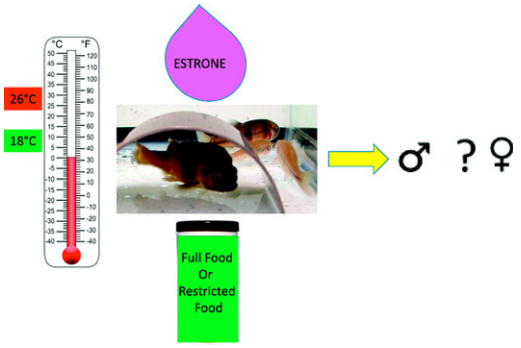
HIGHLIGHTS

- Fish exposure to estrogens occurs under variable temperature and nutrient conditions
- Fathead minnows were exposed to estrone (E1) at 18° and 26 °C, ± food restriction
- Temperature effect dominated, with lower testis weight and vitellogenin at 26 °C
- E1 interactions found for temperature/food for hematocrit, liver size and maturity
- 18 °C most relevant temperature to study endocrine disruption in fathead minnows

GRAPHICAL ABSTRACT



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ABSTRACT

Fish are subject to constantly changing environmental conditions and food availability, factors that may impact their response to endocrine disruptors (EDs). This may, in part, explain outcome discrepancies between field studies and laboratory exposures to EDs. This study assessed whether standard laboratory conditions for fish exposures adequately represent effects of ED exposure at two environmentally realistic temperatures. The impact of temperature and food availability on male fathead minnow response to estrone (E1) exposure was studied in two experiments (3 × 2 × 2 factorial design) with three E1 concentrations (range 0–135 ng/L); two temperatures (18 °C and 26 °C, the latter the prescribed laboratory temperature), and two feeding treatments (full fed vs. 25% of full fed) in a 21-day flow-through system. Morphometric endpoints [including body condition factor, somatic index of gonad (GSI) and liver (HSI), and secondary sex characteristics (SSC)], blood parameters [hematocrit (HCT), blood glucose, cortisol, and vitellogenin (VTG) concentrations], and histology of liver and testis were determined on day 22. High E1 consistently increased VTG, though interactions among E1, temperature and/or food on liver weight, HSI, and HCT were inconsistent between experiments. High temperature impacted the greatest number of parameters, independent of E1 treatment. Three sex-linked parameters were lower at high temperature (testis weight, GSI and VTG), and in Exp. 2SSC and gonad maturity rating were lower. At 26 °C, in Exp. 1 HSI and HCT decreased, and in Exp. 2 length, body and liver weight, and body condition factor were lower. Food

Abbreviations: (E1), estrone; (EtOH), ethanol; (ff), full fed; (GMR), gonad maturity rating; (GSI), gonadosomatic index; (HCT), hematocrit; (HSI), hepatosomatic index; (VTG), vitellogenin.

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restriction decreased GSI in Exp. 1, and blood glucose and liver weight in Exp. 2. At 26 °C several parameters were altered independent of E1 exposure, including three out of four measurements of sperm differentiation. Concordance between laboratory and field investigations of the biological effects of EDs may improve if environmentally-relevant exposure conditions, especially temperature, are employed.

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1. Introduction

In nature, fish are subject to constantly changing environmental conditions and limitations on food availability, potentially impacting their response to endocrine disruptors. Outcome discrepancies between field studies and laboratory exposures to endocrine disruptors may be a result of these conditions. In contrast, toxicity testing is often intentionally over-simplified, as consistent methodology allows for more legitimate comparison of results from different laboratories. For example, culture guidelines set forth by regulatory agencies have generally recommended that water quality parameters, including water temperature be kept constant (Table 1.2, Denny, 1987; U.S. Environmental Protection Agency, 1988). These studies have provided the framework for aquatic toxicologists to develop a vast ecotoxicological database of knowledge. There are, however, limitations to this reductionist approach. Fish populations are not genetically identical (Wang et al., 2016). Variability of individual organismal biology within treatments will inevitably lead to variation in dose responses. Other factors, including seasonal influences on fish reproduction (Denton and Yousef, 1975; Smith, 1978), further emphasize the limitations of standardized exposures. It is important to remember that the ultimate goal of data derived from toxicity testing is to create environmental standards that typically will be applied across habitats encompassing a broad geographical or climatic range. In the case of the fathead minnow (*Pimephales promelas*), a common laboratory model species in aquatic toxicology, spawning may begin as early as March at the edge of the southern-most habitat range, but may not reach peak reproductive season until June or July along its northern range in the Upper Midwest of the USA and Canada. Average temperatures for April and July are presented along with fathead minnow habitat in Fig. 1. The aim of the current study was, therefore, to assess whether standard laboratory conditions for fish exposures adequately represent effects of ED exposure at two environmentally realistic temperatures, 18 °C and 26 °C. Based on water temperature data throughout the range of the fathead minnow in the U.S. (Fig. 1) during peak spawning season (Gale and Bunyak, 1982; Smith, 1978), an environmentally relevant test temperature is approximately 18 °C.

By many accounts, climate change is accelerating, and estimations predict that this trajectory will persist (Murdoch et al., 2000; Adrian et al., 2009). It is expected that changes in gill ventilation and physiology (Evans, 1987; Blewett et al., 2012; Roberts, 2012), elevated metabolic rate (Evans and Claiborne, 2006), and increased estrogen receptor sensitivity (Blair et al., 2000), which occur at higher temperatures, may affect the outcome of E1 exposure in fish. In addition to variability of abiotic environmental conditions, food availability represents another point of departure between laboratory exposure conditions and realities in the environment. While ad libitum feeding is recommended for most toxicity testing, fish in aquatic ecosystems seldom have this luxury for extended periods of time and more likely face prolonged periods of limited food quality and quantity. Biological markers of estrogenic exposure might be enhanced in fish fed with an abundance of food when compared to those with limited food access. The synthesis of VTG in male fish, a well-established biomarker of estrogenic exposure (Sumpter and Jobling, 1995; Ankley et al., 2001; Matozzo et al., 2008; Bartell and Schoenfuss, 2012) may be limited by nutrient availability or may come at the expense of reduced gamete production under limited food availability. Such responses would not be observed in typical laboratory exposures, where food access is ad libitum. If so, detection of estrogenic endocrine disruption would be hindered in laboratory exposures using standard laboratory exposure conditions and the biomarker, VTG.

The overall objective of the current study was to examine the modulating quality of an abiotic (temperature) and biotic (food availability) factor on E1 exposure effects in male fathead minnows. Specifically, we tested the hypothesis that male fathead minnows exposed to E1 at higher temperatures and limited food will exhibit greater adverse effects of exposure than those at any other tested combination of temperature and food availability.

2. Materials and methods

2.1. Experimental design

Two successive 21-d flow-through exposure experiments were conducted at the Aquatic Toxicology Laboratory in St. Cloud, MN (Fig. S1). Previously published flow-through exposure protocols (Schoenfuss et al., 2008) were utilized and modified to reflect the three E1 concentrations, two temperature and two feeding regimes. Treatment groups consisted of two tanks with 10 to 12 mature (six-month-old) male fathead minnows per tank. Estrone concentrations (0, low, and high) were chosen to reflect environmental concentrations in surface waters as previously summarized from the literature (~20–120 ng/L, Fig. 1 of Dammann et al., 2011; Ankley et al., 2017). Based on the available literature, our low E1 treatment concentrations are found frequently in environments with anthropogenic inputs, while the high E1 treatment concentrations in this study reflect worst case environmental conditions, or may represent the totality of estrogenic activity measured in a body of water (often expressed as Estradiol Equivalency Quotient – EEQ; for example, Schultz et al., 2013). Each estrone treatment concentration (0, low and high) was delivered from a common mixing tank to assure that all aquaria in the same treatment received the same E1 concentration (Water flow diagram, SI Fig. 1). Estrone treatments were further replicated under two different temperatures (low ~18 °C and high ~26 °C) and feeding conditions (restricted to 0.75% body weight/d [25% ff] or full-fed at 3% body weight [ff]). On day 22 of the exposure, all fish were assessed for morphological characteristics (length, weight, body condition factor, secondary sex characteristics [SSC], hepatosomatic index [HSI] and gonadosomatic index [GSI]). Blood and tissue samples were collected for analysis of physiological biomarkers (hematocrit [HCT], blood glucose, plasma VTG and cortisol concentrations [0 and high E1 treatments only]) and histological endpoints.

2.2. Exposure chemicals

Estrone (Reference standard,Sigma-Aldrich, St. Louis, MO) exposure solutions were prepared daily and delivered as previously described (companion paper, Part 1.). Analysis of stock estrone solutions in ethanol diluted to ~100 pg/μL and analyzed by LCMSMS detected no estriol, 17α- or β-estradiol (detection limits 1 pg/ul on column). Water exchange rate was approximately seven exchanges/aquarium/d.

2.3. Water

2.3.1. Water quality

Water temperature was monitored continuously using a HOBO Data Logger (Onset Computer Corporation, Bourne, MA). Nominal temperatures were 18 °C and 26 °C, with measured mean low temperature for

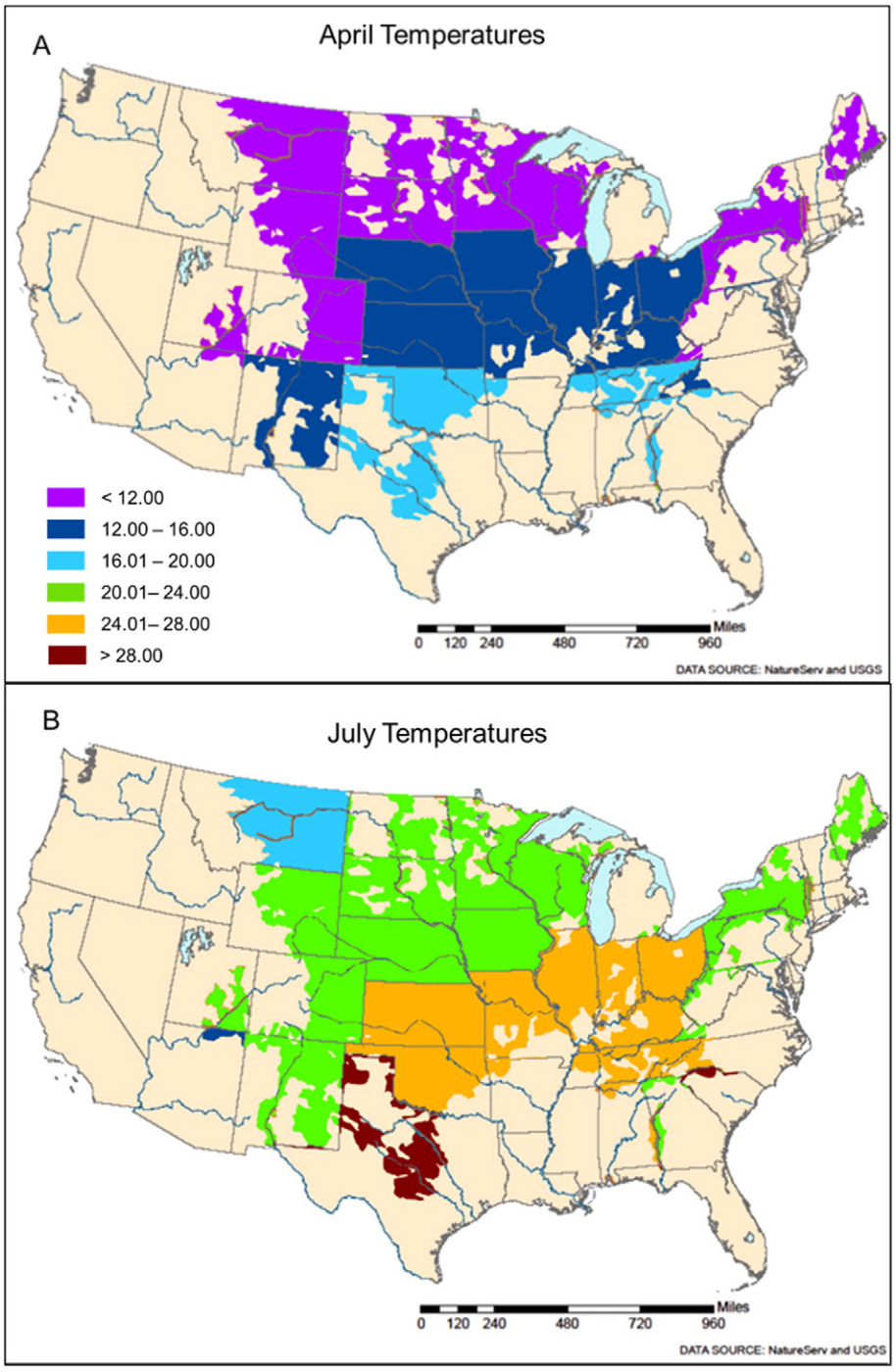

Fig. 1. Native fathead minnow habitat with average temperatures (°C) in April (A) and July (B), reflecting early spawning periods for southern and northern spawning regions of United States.

both experiments of 18 °C (17.6 °C Exp. 1, 17.8 °C experiment 2, COVs ≤ 3%), and high temperatures of 26.8 °C and 25.6 °C for Exps. 1 and 2, respectively (COVs = 2%). As a failsafe, additional daily water temperatures were taken along with pH (General PH-501, General Tools & Instruments, New York, NY) and conductivity measurements (General CO-502, General Tools & Instruments, New York, NY). Total water hardness, free chlorine, total chlorine and alkalinity were periodically assessed (~every 3 d) using AquaChek 5-in-1 Water Quality Test Strips (Hach Company, Loveland, CO).

2.3.2. Water chemistry

Mixing tank water samples on exposure d 5, 10, 15, and 20, were collected from solvent (EtOH) controls and both low and high E1 treatments held at 18 °C or 26 °C. Water samples were stored at −20 °C for 24 h, then transferred to −80 °C for storage until analysis. Because of the high mineral content of the well water, samples required extraction on OASIS solid phase columns prior to LC MS^2 analyses using previously published methods (Dammann et al., 2011). While the mean E1 treatment concentrations in Exp. 1 were lower than in Exp. 2 (low: 9

± 1.6 ng/L, high: 78 ± 22.9 ng/L vs. low: 14 ± 2.1 ng/L, high: 135 ± 20.7 ng/L), high E1 concentrations were ~9 times the low E1 in both experiments.

2.4. Fish

Adult male fathead minnows were obtained from a laboratory fathead minnow culture (US EPA Cincinnati) and acclimated to on-site physico-chemical conditions for at least 14 d prior to the start of the experiment. Fish were maintained at ~22 °C and ad libitum fed during the acclimation period using a similar combination of blood worms and brine shrimp as during the latter experiments. Approximately 48 h prior to the commencement of E1 exposure, water temperature was slowly lowered or increased to acclimate fish to the 18°C and 26°C exposure temperatures, respectively. Mortality was assessed daily. Fish were maintained throughout the exposure experiments at one of two constant water temperatures (18 °C and ~26 °C). A mixture of brine shrimp (*Artemia* spp., Brine Shrimp Direct, Ogden, UT) and blood worms (*Glycera* spp.) was fed daily at 3% of fish body weight (using an average male fathead minnow weight of 3.3 g) for the full fed treatment (ff), or 0.75% of body weight for the limited food treatments (25% ff). No adjustments in food portions were made over the course of the experiment. Fish were maintained and sacrificed in accordance with St. Cloud State University's IACUC policies (Protocol # 06-09).

2.5. Biological endpoints

2.5.1. Organosomatic indices

Fish length, body, liver and testis organ weight, hepatic somatic index, and gonadosomatic index were determined as previously described (companion paper). Briefly, organ wet weight was divided by fish wet weight and multiplied by 100 to obtain a relative weight of the organ.

2.5.2. Secondary sex characteristics

As an indication of sexual maturity, prior to dissection, a visual assessment of the male sexual characteristics of individual fish was performed. Tubercles, dorsal pad and banded coloration were each visually scored by laboratory staff at the time of fish dissection, and given a score of 1, 2, or 3, with 1 being the least prominent and 3 being the most prominent. The three values were analyzed separately, and as a single composite value as a method for comparing sexual maturity between treatments.

2.5.3. Blood parameters

Blood was collected in microcapillary tubes from the caudal vasculature after tail severance. Microcapillary tubes were centrifuged and the HCT determined (see Feifarek et al., accepted for detailed methodology). Blood glucose was measured using a clinical blood glucose meter (TRUEbalance Blood Glucose Monitor, Moore Medical, Farmington, CT). Plasma VTG concentrations (μg/mL) were quantified via competitive antibody-capture ELISA following established protocols (Dammann et al., 2011) and as previously described in detail (companion paper). Cortisol concentrations (pg/mL) were measured on control and high E1 treatment fish only, using a cortisol enzyme immunoassay (EIA) (Cortisol Express EIA Kit # 500370, Cayman Chemical Company, Ann Arbor, MI) following instructions provided with the kit. Plasma was diluted 1:40 and 1:80, and five microliters of each dilution assayed. Plates were washed using a Stat Fax 2600 Microplate Washer (Awareness Technology, Palm City, FL), absorbance measured on a Multiskan EX spectrophotometer (Thermo Scientific, Waltham, MA) and data analyzed using its Ascent Software.

2.5.4. Histopathology

Liver and testis histology and scoring for assessment of liver cellular vacuolization and gonad maturity were performed as previously described (companion paper). Vacuolization scores ranged from 1 - no vacuoles, to 4 – vacuolization across most of the tissue slice. To assess the overall maturity of the testis, a modified approach to Vajda et al. (2008) was employed. The percent of spermatogonia, spermatocytes, spermatids, and spermatozoa present in the tissue were used to calculate the testis maturity status representing overall spermatogenesis.

$$\text{Gonad maturity rating (GMR)} = ((\%_{\text{spermatogonia}}) + (\%_{\text{spermatocytes}} \times 2) + (\%_{\text{spermatids}} \times 3) + (\%_{\text{spermatozoa}} \times 4))/100$$

2.5.5. Statistical analysis

The effects of E1 (control, low, high), water temperature (18 °C, ~26 °C) and feed (25% and 100% full feed, respectively) were assessed using a three-way analysis of variance (ANOVA). In the models, tanks were considered the sampling unit. Secondary sex characteristics were modeled using the Poisson distribution. Percent spermatogonia, spermatocytes, spermatids and spermatozoa were transformed using the arc tangent transformation prior to analysis. If significant interactions were found in the ANOVAs, Tukey's contrasts were used for pair-wise comparisons. Means for control values of some parameters appeared to differ by experiment, therefore data was subjected to a two-way ANOVA to test for differences between experiments by temperature.

Table 1
Experiment 1. Effects of estrogen, temperature, and feed availability on survival and somatic scores in fathead minnows (mean ± standard error); na not applicable; ns not significant; FF full feed; superscripts indicate statistically differences.

Experiment 1			Survival %	Body condition factor (BCF)	Hepatosomatic index (HSI)	Liver vacuolization	Hematocrit %	Blood glucose (μg/mL)	Cortisol (mg/dL)
ANOVA			na	ns	Temp $P = 0.02$ 18 °C 1.1 ± 0.06≠ 27 °C 0.9 ± 0.06	ns	E1xTempxFood $P = 0.03$	ns	ns
Control	18 °C	25%FF	100	1.10 ± 0.05	1.06 ± 0.15	2.46 ± 0.50	35.4 ± 5.1^{ab}	21.5 ± 10	36.2 ± 11
		FF	100	1.08 ± 0.05	1.18 ± 0.15	2.37 ± 0.45	38.2 ± 5.1^{ab}	31.0 ± 10	38.5 ± 11
	27 °C	25%FF	75	1.06 ± 0.05	0.99 ± 0.15	2.88 ± 0.59	44.1 ± 5.1^{abc}	34.1 ± 10	41.7 ± 11
		FF	95	1.02 ± 0.05	0.98 ± 0.15	2.69 ± 0.51	47.7 ± 5.1^{abc}	41.1 ± 10	33.9 ± 11
Low E1	18 °C	25%FF	100	1.04 ± 0.05	1.07 ± 0.15	2.11 ± 0.44	63.4 ± 5.1^{b}	36.0 ± 10	na
		FF	100	1.06 ± 0.05	1.15 ± 0.15	2.02 ± 0.40	52.5 ± 5.1^{abc}	29.3 ± 10	na
	27 °C	25%FF	70	1.03 ± 0.05	0.78 ± 0.15	2.10 ± 0.45	32.7 ± 5.1^{ab}	29.8 ± 10	na
		FF	100	1.08 ± 0.05	1.05 ± 0.15	2.50 ± 0.49	43.3 ± 5.1^{abc}	51.3 ± 10	na
High E1	18 °C	25%FF	100	1.15 ± 0.05	1.10 ± 0.15	2.11 ± 0.44	48.6 ± 5.1^{abc}	42.3 ± 10	49.5 ± 11
		FF	100	1.05 ± 0.05	1.30 ± 0.15	2.62 ± 0.49	67.8 ± 5.1^{c}	54.6 ± 10	34.3 ± 11
	27 °C	25%FF	90	1.04 ± 0.05	0.86 ± 0.15	2.57 ± 0.50	43.7 ± 5.1^{abc}	42.5 ± 10	41.1 ± 11
		FF	90	1.02 ± 0.05	0.84 ± 0.15	2.56 ± 0.49	38.5 ± 5.1^{ab}	48.8 ± 10	51.6 ± 11

Table 2
Experiment 2. Effects of estrogen, temperature, and feed availability on survival and somatic scores in fathead minnows (mean ± standard error); na not applicable; ns not significant; FF full feed.

Experiment 2			Survival %	Body condition factor (BCF)	Hepatosomatic index (HSI)	Liver vacuolization	Hematocrit %	Blood glucose (μg/mL)	Cortisol (mg/dL)
ANOVA			na	Temp $P < 0.001$ 18 °C 1.17 ± 0.02≠ 26 °C 1.02 ± 0.02	Food $P = 0.02$ E1xTemp $P = 0.01$ Control 26 °C ≠ High E1 18 °C 1.0 ± 0.10≠ 1.4 ± 0.08 High E1 18 °C ≠ High E1 26 °C 1.4 ± 0.08≠ 1.0 ± 0.08 $P = 0.05$	E1 ChiSq $P = 0.04$ by E1 individual	TempxFood $P = 0.02$ ns by group 18 °C 52 ± 3≠ 26 °C 40 ± 3 $P = 0.08$	Food $P = 0.02$ 25%FF 60 ± 5.8≠ FF 84 ± 6.3	ns
Control	18 °C	25%FF	100	1.17 ± 0.04	1.12 ± 0.11	2.26 ± 0.35	44.8 ± 5.1	73.0 ± 14.2	17.7 ± 6.2
		FF	100	1.14 ± 0.04	1.12 ± 0.11	2.50 ± 0.35	46.2 ± 5.1	89.1 ± 14.2	13.2 ± 6.3
	26 °C	25%FF	95	0.96 ± 0.04	0.87 ± 0.11	1.94 ± 0.34	55.5 ± 5.1	69.1 ± 14.2	16.2 ± 6.2
		FF	65	1.01 ± 0.06	1.08 ± 0.16	2.00 ± 0.58	39.5 ± 7.2	71.5 ± 20.1	22.6 ± 8.8
Low E1	18 °C	25%FF	100	1.20 ± 0.04	1.10 ± 0.11	2.58 ± 0.37	38.6 ± 5.1	55.0 ± 14.2	na
		FF	100	1.20 ± 0.04	1.10 ± 0.11	2.94 ± 0.40	46.7 ± 5.1	98.4 ± 14.2	na
	26 °C	25%FF	90	1.06 ± 0.04	1.17 ± 0.11	2.70 ± 0.40	39.8 ± 5.1	53.1 ± 14.2	na
		FF	90	1.09 ± 0.04	1.48 ± 0.11	2.92 ± 0.47	43.4 ± 5.1	69.4 ± 14.2	na
High E1	18 °C	25%FF	100	1.11 ± 0.04	1.18 ± 0.11	2.25 ± 0.34	42.5 ± 5.1	55.2 ± 14.2	16.1 ± 6.2
		FF	100	1.18 ± 0.04	1.60 ± 0.11	2.35 ± 0.34	64.2 ± 5.1	106.6 ± 14.2	20.3 ± 6.2
	26 °C	25%FF	85	0.95 ± 0.04	0.93 ± 0.11	1.73 ± 0.40	45.6 ± 5.1	57.4 ± 14.2	27.1 ± 6.2
		FF	85	1.03 ± 0.04	1.10 ± 1.11	2.44 ± 0.39	38.1 ± 5.1	72.2 ± 14.2	20.6. ± 6.2

Controls were found to differ by experiment ($P \leq 0.05$), and subsequently all data from each experiment was analyzed separately.

3. Results and discussion

Contrary to our a priori hypothesis that E1 exposure would interact with temperature and feed limitation to change the biological responses to E1 exposure, we instead identified temperature as the key driver of changes in biomarker expression among treatments. Consequently, the observed effects are principally reported and discussed in the context of temperature rather than E1 effects. The prevalence of temperature-dependent changes in biomarker expression validates the aim of the current study to assess whether standard laboratory conditions for fish exposures adequately represent effects of ED exposure at two environmentally realistic temperatures.

3.1. Survival

High temperature increased mortality, independent of other treatments, in both Exp. 1 and 2 (% survival by tank, mean of two tanks, (Tables 1 & 2). Statistical analysis of temperature effect was not conducted due to 100% survival at 18 °C in Exp. 1 (no variability, Exp. 2

Table 3
Experiment 1. Parameters historically affected by estrogen exposure in fathead minnows (mean ± standard error); na not applicable; ns not significant; FF full feed.

Experiment 1			Secondary sex characteristics (SSC)	Gonadosomatic index (GSI)	Gonad maturity ranking (GMR)	Vitellogenin (VTG) (ng/mL)	Testis weight (mg)
ANOVA			ns	Temp $P = 0.001$ 18C1.1 ± 0.06≠ 26C 0.9 ± 0.04 Food $P = 0.05$ 25%FF 1.1 ± 0.09≠ FF 1.0 ± 0.06	ns	E1 $P < 0.0001$ Con ≠ Hi E1 LoE1 ≠ Hi E1 160 ± 28 (Con) 332 ± 63 (Low E1) 2094 ± 409 (Hi E1) *Con ≈ LoE1 0.08* Temp $P = 0.004$ 18 °C 1194 ± 391≠ 26 °C 530 ± 198	E1 $F_{2,12} = 5.9$ $P = 0.02$ Con 26 ± 15.5, Low E1 26 ± 15.5 ≠Hi E1 33 ± 15.5 Temp $F_{1,12}$ 9.2 $P = 0.01$ 18 °C 31 ± 1.3≠ 27 °C 26 ± 1.3
Control	18 °C	25%FF	5.15 ± 0.58	1.11 ± 0.06	3.23 ± 0.18	272 ± 50	28.2 ± 30.9
		FF	5.82 ± 0.60	0.99 ± 0.12	3.30 ± 0.18	158 ± 6.5	27.4 ± 30.9
	27 °C	25%FF	4.27 ± 0.58	1.02 ± 0.13	3.82 ± 0.18	92.0 ± 2.5	25.6 ± 30.9
		FF	5.24 ± 0.59	0.77 ± 0.04	3.50 ± 0.18	118 ± 19.8	22.6 ± 30.9
Low E1	18 °C	25%FF	4.53 ± 0.56	1.15 ± 0.00	3.02 ± 0.18	351 ± 94.4	28.5 ± 30.9
		FF	5.58 ± 0.56	0.97 ± 0.06	3.43 ± 0.18	465 ± 214	26.0 ± 30.9
	27 °C	25%FF	4.77 ± 0.58	0.78 ± 0.04	3.60 ± 0.18	295 ± 138	20.7 ± 30.9
		FF	5.88 ± 0.64	1.01 ± 0.05	3.60 ± 0.187	216 ± 64	29.6 ± 30.9
High E1	18 °C	25%FF	6.32 ± 0.66	1.41 ± 0.09	3.62 ± 0.18	3492 ± 89	43.0 ± 30.9
		FF	5.60 ± 0.58	1.18 ± 0.11	3.38 ± 0.18	2429 ± 172	32.5 ± 30.9
	27 °C	25%FF	5.16 ± 0.60	0.98 ± 0.11	3.46 ± 0.18	691 ± 160	28.2 ± 30.9
		FF	4.99 ± 0.57	0.88 ± 0.12	3.10 ± 0.18	1765 ± 687	26.6 ± 30.9

Table 4
Experiment 2. Parameters historically affected by estrogen exposure in fathead minnows (mean ± standard error); na not applicable; ns not significant; FF full feed.

Experiment 2			Secondary Sex Characteristics (SSC)	Gonadosomatic Index (GSI)	Gonad Maturity Ranking (GMR)	Vitellogenin (VTG) (ng/mL)	Testis Weight (mg)
ANOVA			Temp ChiSq $P < 0.003$ 18 °C 6.9 ± 0.30≠ 26 °C 5.6 ± 0.32	Temp $F_{1,12} = 20.4$ $P = 0.001$ 18 °C 1.4 ± 0.08≠ 26 °C 0.7 ± 0.09	Temp $F_{1,11} = 12.0$ $P = 0.005$ 18 °C 3.5 ± 0.11≠ 26 °C 2.9 ± 0.12	E1 $P < 0.0001$ Con ≠ High E1 LoE1 ≠ High E1 126 ± 29 (Con) 170 ± 28 (Low E1) 1697 ± 351 (HighE1) Temp $P = 0.003$ 18 °C 500 ± 185≠ 26 °C 892 ± 357	Temp $F_{1,11} = 47.3$ $P < 0.0001$ 18 °C 47.8 ± 2.7≠ 26 °C 20.4 ± 2.9
Control	18 °C	25%FF	7.29 ± 0.76	1.49 ± 0.19	3.62 ± 0.26	77 ± 12.1	50.3 ± 6.6
		FF	7.25 ± 0.75	1.24 ± 0.19	3.28 ± 0.26	63 ± 4.5	44.9 ± 6.6
	26 °C	25%FF	5.21 ± 0.62	0.59 ± 0.19	2.72 ± 0.26	187 ± 45	13.6 ± 6.6
		FF	4.83 ± 1.00	0.57 ± 0.28	2.68 ± 0.37	225 $n = 1$	19.9 ± 9.4
Low E1	18 °C	25%FF	6.72 ± 0.73	1.17 ± 0.19	3.40 ± 0.26	101 ± 16.6	40.4 ± 6.6
		FF	7.27 ± 0.77	1.36 ± 0.19	3.42 ± 0.26	121 ± 27.2	51.0 ± 6.6
	26 °C	25%FF	5.95 ± 0.70	0.85 ± 0.19	3.32 ± 0.26	233 ± 80	25.1 ± 6.6
		FF	6.33 ± 0.81	0.76 ± 0.19	2.68 ± 0.26	225 ± 31.1	20.5 ± 6.6
High E1	18 °C	25%FF	6.75 ± 0.72	1.39 ± 0.19	3.60 ± 0.26	1024 ± 260	46.0 ± 6.6
		FF	6.35 ± 0.69	1.55 ± 0.19	3.53 ± 0.26	1617 ± 57	54.0 ± 6.6
	26 °C	25%FF	5.75 ± 0.82	0.81 ± 0.19	3.02 ± 0.26	2527 ± 1367	22.4 ± 6.6
		FF	5.43 ± 0.68	0.87 ± 0.19	3.15 ± 0.26	1621 ± 592	21.0 ± 6.6

mean survival per tank was 99% ± 3% S.D., Table S1). At ~26 °C, in Exp. 1, mean survival was 87% ± 15%, and 89% ± 5% in Exp. 2. By comparison, survival values for fathead minnows in this laboratory typically average 95%. A temperature of approximately 18 °C is often considered an optimum breeding temperature for natural populations of fathead minnows across most of their range. The highest mortality (60%) resulted due to a technical malfunction when water flow into one tank was blocked by occluded tubing (Exp. 2, Control, 26 °C, ff). Consequently, the data from fish in that tank were excluded from statistical analyses. Increased mortality with higher water temperatures considered in the context of climate change-induced increases in surface water temperature may foreshadow serious ramifications at the population level (Pörtner and Knust, 2007; Noyes et al., 2009).

3.2. Biological endpoints typically altered by E1

3.2.1. Secondary sex characteristics

Of parameters expected to be affected by E1 exposure, no effect was found for SSC, GSI, or GMR in either experiment. While there have been previous reports of decreased SSC with estrone exposure (Dammann et al., 2011), similar to findings in the companion paper, estrone exposure did not affect SSC in either experiment (Tables 3 & 4, SI 2). In

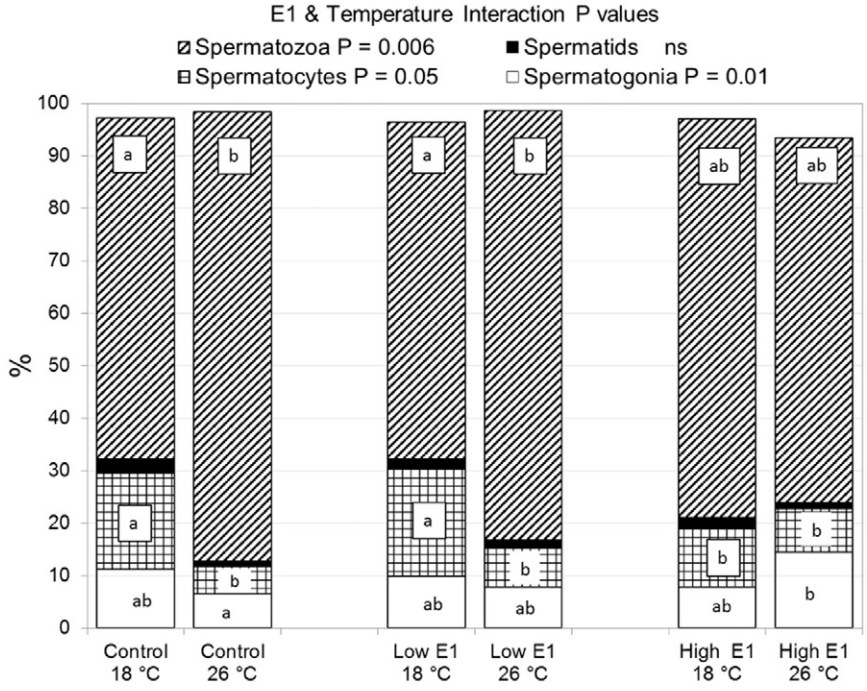

Fig. 2. Sperm maturation status, Exp. 1, from spermatogonia to spermatozoa, as a percent of total. Significant E1 and temperature interactions are as indicated ($P \leq 0.05$ with exception of spermatogonia, $P = 0.06$) with differences within sperm state due to treatment indicated by different letters. No significance (ns). Because data were not normally distributed, analysis required logit transformation, and therefore the sum of the means does not add up to exactly 100%.

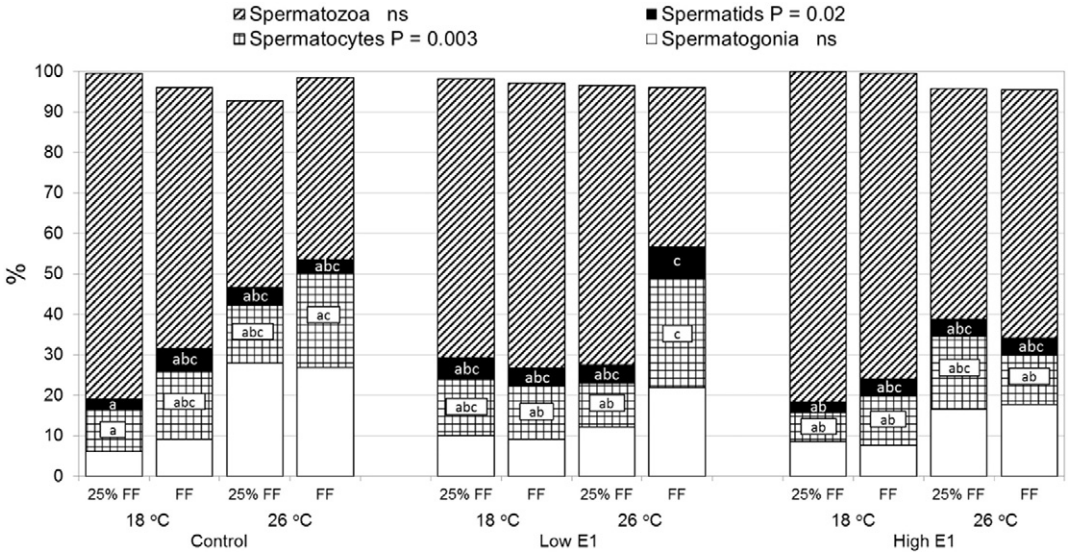

Fig. 3. Sperm maturation status, Exp. 2, from spermatogonia to spermatozoa, as a percent of total. Significant E1, temperature, and feed interactions are as indicated, differences within sperm state are indicated by different letters. No significance (ns). Because data were not normally distributed, analysis required logit transformation, and therefore the sum of the means does not add up to exactly 100%.

contrast, temperature decreased SSC at 26 °C in Exp. 2 (5.6 vs. 6.9. at 18 °C, $P < 0.003$, Table 4). Absence of a temperature effect in Exp. 1, might be explained by the lower control values at 18 °C (mean across both feeding treatments, 5.5 ± 0.42 in Exp. 1 vs. 7.3 ± 0.53 in Exp. 2, data not shown).

3.2.2. GSI and testis weights

Similar to SSC, a decrease in GSI was found with higher temperature and in both experiments. Mean GSI was reduced by 20% in Exp. 1 (Table 3) and 46% in Exp. 2 (Table 4). Because food availability could affect body weight, GSI would not necessarily indicate a decrease in testis

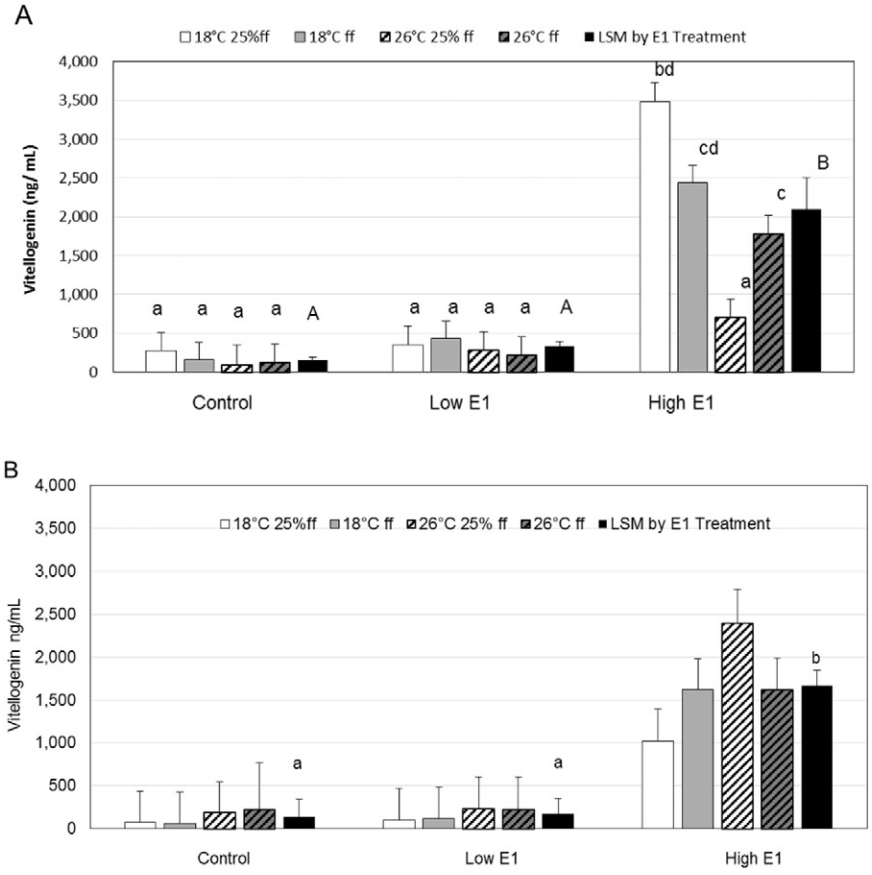

Fig. 4. Plasma vitellogenin. Treatments abbreviations defined in Fig. 2. Mean values ± S.E. from Exp. 1 (A) and Exp. 2 (B). Least square means (LSM) are presented for control, low E1, and high E1 treatments with different letters indicate differences ($P < 0.0001$).

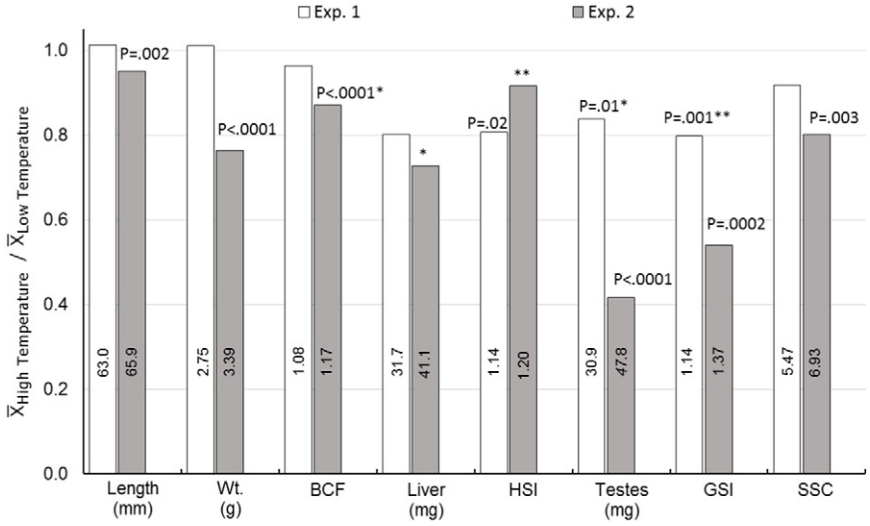

Fig. 5. Effect of water temperature on morphometric parameters. Ratio of Parameter $_{high\ temperature}$/Parameter Mean $_{low\ temperature}$, for Exp. 1 & 2. Mean value of parameter at 18 °C within bar. Level of statistical signficance between high and low temperature means indicated as P value above bars. * indicates other statistical main effect differences, or interactions.

weight. Therefore, both body and testis weights were analyzed separately (SI Table 3–5 and Tables 3 & 4). Testis weight in Exp. 1 was altered by both estrone ($P = 0.02$) and temperature ($P = 0.01$). High estrone caused an increase in testis weight (32.6 ± 1.5 mg versus ~26 mg for control or low estrone treatments, Table 4). Elevated temperature was associated with decreased testis weight in both experiments (decrease of 17%, $P = 0.01$ in Exp. 1; and 57%, $P < 0.001$ in Exp. 2).

3.2.3. Comparison of gonad maturity rating and individual sperm stages

While gonad maturity rating (GMR) was not affected by estrone (SI Table S2), a decrease in GMR was seen with higher temperature in Exp. 2 (15% decrease, $P = 0.005$, Table 4). Comparing differences in individual stages of sperm development (SI Table 6), temperature had an effect on 3 of the 4 stages of sperm in both experiments, though interactions with environmental treatment differed. In Exp. 1, the interaction was between E1 treatment and temperature ($P \leq 0.05$) for all cell stages except spermatids. Fig. 2 represents the cross sections of testis as populated by maturing sperm. High temperatures were associated with an increase in the percent of mature sperm (spermatozoa) and respective decrease in spermatocytes in control and low E1 treatments. These findings are in agreement with a previous report of higher temperature associated with higher abundance of mature sperm (Smith, 1978). While temperature effects were eliminated in the presence of high E1 treatments, the percent spermatogonia at 27 °C and high E1 was higher than at 27 °C for control fish (14.4 ± 1.9% vs. 6.5 ± 1.4%).

In contrast to Exp. 1, in Exp. 2 (Fig. 3), interactions included effect of food availability (three way) for spermatocytes and spermatids ($P \leq 0.02$), and spermatozoa ($P = 0.06$). The only statistical differences by treatment were found in spermatocytes and spermatids, which were both lower in fish from control 18 °C 25% ff than fish from the low E1, 27 °C ff treatment. While treatment interactions for spermatogonia were not significant, temperature effect was the opposite as found in Exp. 1, higher temperature appeared to "stall" cells as spermatogonia was (18 °C 8.4 ± 1.5% versus 27 °C 20 ± 2.4%, $P = 0.002$). Statistically, high E1 treatment ablated differential temperature responses across all stages of sperm development. Because fish were sacrificed after only one exposure period, it is unclear if higher E1 concentrations (135 ng/L) arrest sperm maturation, or stimulate spermatogonia proliferation.

Finding an increase in percent spermatogonia with estrogen treatment is in agreement with in vitro studies of testicular explants and primary cell culture. These studies reported estrogens increase spermatogonia (17β-estradiol in eels [*Anguilla japonica*, Miura et al., 1999] and in Japanese huchen [*Hucho perryi*, Amer et al., 2001]; 17α-ethinylestradiol in medaka [*Oryzias latipes*, Song and Gutzeit, 2003]). Sperm maturation was arrested in the presence of E2, but resumed after an E2 depuration period (Miura et al., 1999). Similarly, testis maturation scores of medaka decreased when exposed to 17α-ethinylestradiol for 90 d post-fertilization (Luzio et al., 2016), yet recovered with a 30 d depuration period. In contrast, when sperm cells of rainbow trout (*Oncorhynchus mykiss*) sorted by state of maturation were treated with similar 17β-estradiol concentrations, no significant increase in proliferation occurred (Loir, 1999). It is unclear if this was due to differences in methodology or species.

3.2.4. Plasma vitellogenin

Vitellogenin, the most predictable indicator of estrogenic exposure in male FHM, was significantly affected by both E1 ($P < 0.0001$) and temperature ($P \leq 0.004$) in both experiments (Fig. 4, Tables 3 & 4). Estrone did not significantly elevate plasma VTG concentrations in fish exposed to low E1 concentrations (9 or 14 ng E1/L, Exps. 1 and 2, respectively) at any temperature or feeding regimen. Failure of E1 concentrations ≤14 ng/L to significantly increase plasma VTG affirms previous reports (10 ng/L Panter et al., 1998, 15 ng/L Dammann et al., 2011, and 18 ng/L companion paper). As expected, higher E1 concentrations resulted in higher plasma VTG.

In Exp. 1, high E1 treatment of fish on food restriction held at 27 °C failed to have significantly elevated VTG ($P = 0.82$). This was in accord with initial thoughts that limited nutrient availability in the presence of a temperature-induced increase in metabolism might reduce VTG production. It may be that in order to maintain homeostasis under conditions associated with a higher metabolic rate, reproductive functions will be sacrificed (particularly energy-intensive functions such as vitellogenesis), in order to survive (Luquet and Watanabe, 1986; Izquierdo et al., 2001). In such circumstances, evidence of endocrine disruption could be obscured, due to lack of the hallmark elevated VTG. The lack of VTG response with 25% ff, high temperature and E1 concentrations was not seen in Exp. 2, perhaps a reflection of the higher E1 concentrations in Exp. 2. Moffatt et al. (2010) concluded that 72 h of food restriction failed to statistically decrease VTG mRNA expression (vs. unrestricted food) in medaka exposed to 272 ng/L 17β-estradiol. Review of their data indicates a ≥ 50% decrease in ratio of VTG mRNA/ribosomal protein RNA with food restriction compared to fish without food restriction. The food restricted fish in Moffatt et al. (2010) were, however, maintained in a field exposure apparatus within the tank, while fish in the present study were unrestricted within

a tank and assayed in separate experiments. The stress of physical restriction within the apparatus may have resulted in lower VTG mRNA, even with unrestricted food. Another difference between this study and the present one is the period of food restriction, 72 h versus 21 d.

While a statistically significant temperature effect on VTG was seen in both experiments, the increase was associated with opposite temperatures in each experiment. In Exp. 1 the higher temperature resulted in lower log VTG (2.37 ± 0.06 vs. 2.68 ± 0.06), while in Exp. 2 it resulted in higher VTG (2.54 ± 0.06 vs. 2.23 ± 0.06). Again, we hypothesize that the difference is due to the higher E1 concentrations in Exp. 2 (135 vs. 78 ng/L). The higher E1 concentration in Exp. 2 might have been expected to produce a higher VTG response, but this was not the case. This may be due to a diminished dose response to estrogenic chemicals reported previously (Wester et al., 2003; Brian et al., 2008); hepatic and renal toxicity similar to that which has been observed in male summer flounder (*Paralichthys dentatus*) injected with estradiol (Folmar et al., 2001); or a consequence of the high mortality seen with high E1 in Exp. 2 (15%). Surviving individuals with a reduced susceptibility to E1's VTG-inducing effects may have been inadvertently selected for in the sample, resulting in lower mean VTG. While organismal variability exists between fish, perhaps more importantly, seasonal variability plays a role in reproductive function in fathead minnows (McMillan and Smith, 1974; Denton and Yousef, 1975; Smith, 1978). The changing seasons during which the experiments were conducted (September to November) may have played a role in the rate of vitellogenesis, as the timeframe is moving farther away from peak spawning season for fathead minnows (Fig. 1), and the mean VTG concentration was indeed lower in the second experiment (November). It is noteworthy that reduced fecundity and fertility in fall and winter is a phenomenon observed by aquaculturists of laboratory fishes (personal communications documented in SI) and occurs despite the many generations of removal of these fish from natural habitats and despite the 16:8 h light/dark constant photoperiod maintained in most laboratory fish cultures.

When comparing VTG response to high E1 in Exp. 1 and 2, mean VTG were lower in the later experiment. And in contrast to predictions, the highest mean plasma VTG concentration for high E1 in Exp. 1 was found in fish under food restriction and held at 18 °C (3489 ng/mL) though not statistically different from those ff (2438 ng/mL). These values were 13 times the mean plasma VTG concentrations of control fish or 10 times those in low E1 maintained at 18 °C with restricted food. The elevation in VTG may be in response to two factors: (i) enhancement of estrogen-induced vitellogenesis due to the stress hormone cortisol; and (ii) more optimal breeding temperatures near 18 °C triggering reproductive potency. Cortisol (10–20 μg/100 mg body weight) enhances the induction of vitellogenesis via 17β-estradiol in Asian stinging catfish (*Heteropneustes fossilis*) purportedly through peptide hormones and enzyme induction (Sundararaj et al., 1982). Additionally, fathead minnow spawning tends to begin when water temperatures reach about 14 °C to 18 °C (McCarraher and Thomas, 1968) and literature suggests reproductive efficacy peaks early in the spawning season, then tapers off as water temperatures warm (Smith, 1978).

3.3. Biological endpoints not typically altered by E1

3.3.1. Length, body and liver weights, BCF, and HSI

Fig. 5 represents the overall means for morphometric parameters of fish maintained at higher temperature as a percent of those maintained at the lower temperature. Clearly, elevated temperature had an effect on three morphometric parameters in Exp. 1, and six in Exp. 2 - including some interactions with E1 (SI Table 3–5). Liver weights and HSI were the only morphometric variables *sometimes* affected by E1 treatment, but the E1 effects at times included interactions with either temperature or food availability. While in Exp. 1, no main treatment effect or

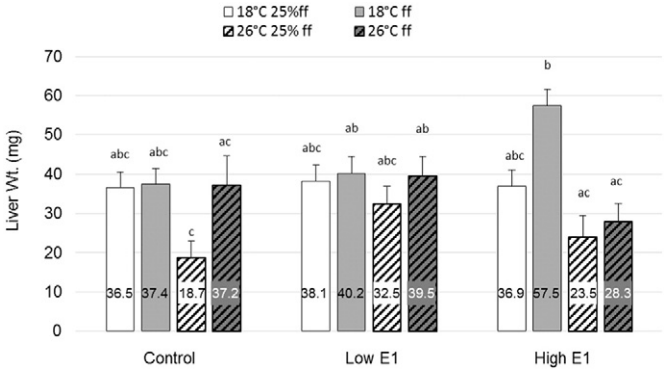

Fig. 6. Three way interaction of treatment effects on mean liver weights in Exp. 2. Treatment definitions as follows: Low ° = 18 °C, High ° = 26 °C, 25% ff = 25% of full fed, ff = full fed. Treatments with common letters are not statistically different ($P \leq 0.05$).

interaction was found on length, body and liver weights at the $P > 0.05$ level (SI Table 5), an effect on liver weights approached significance ($P = 0.06$) with a 20% reduction at higher temperature. In Exp. 2, elevated temperature decreased all three parameters, though a three-way treatment interaction was present on liver weights (Fig. 6, SI Table 5, $P = 0.04$). While food restriction decreased liver weights at 26 °C for control fish, in the presence of high E1, food restriction has no effect on liver weight. In contrast, at 18 °C and high E1, a significant increase in liver weight was seen (57 mg). One would expect a decrease in glycogen stores and therefore liver weights when nutrient availability was decreased and temperature elevated as seen with control fish. The high liver weights found with high E1 exposure were most likely the result of biosynthesis of vitellogenin within the liver tissue. (Tables 3 & 4, Control and high E1 2.17 vs. 2.78 for low E1). Examples of liver vacuolization scoring is provided in SI Fig. 5. Others have suggested increased vacuolization reflects increased nutrient storage (Hoar and Randall, 1988; Dietrich and Krieger, 2009).

3.3.2. Blood parameters: hematocrit, glucose, and cortisol

3.3.2.1. Hematocrit. An estrone x temperature x food interaction was found on hematocrit in Exp. 1 ($P = 0.03$, Fig. 7, Tables 1, 2 and SI Table 7). The highest hematocrit (and notably the highest mean liver weight) occurred in ff fish exposed to high E1 at 18 °C (Fig. 7). Hematocrit of fish exposed to low E1 at 18 °C were higher than control fish at 18 °C. Higher temperatures appeared to ablate any increase in hematocrit due to E1 exposure. A temperature by food interaction was found in Exp. 2 ($P = 0.02$), though differences between groups failed to reach significance. Mean for ff fish at 18 °C tended to be higher than at 26 °C (52 ± 3 vs. 40 ± 3, $P = 0.08$). Looking at hematocrit means across all

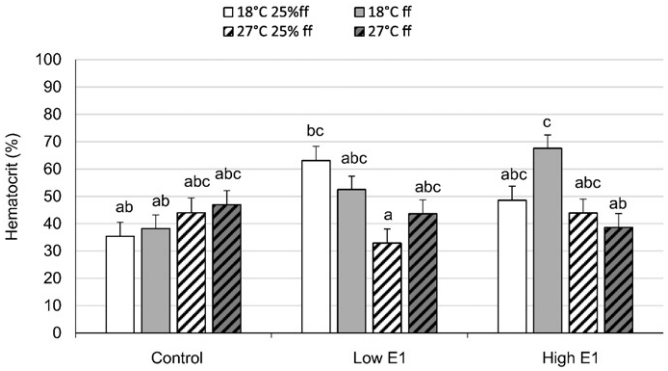

Fig. 7. Hematocrit. Treatments abbreviations defined in Fig. 2. A. Mean values ± S.E. from Exp. 1, different letters indicate differences ($P = 0.03$, mean ± S.D.).

Table 5
Differences for control full feed (FF) fish at 18 °C or 26 °C between Experiment 1 and 2 (mean ± standard error); superscripts indicate statistical differences by experiment at the same temperature, $P \leq 0.02$ (bolded).

Parameters measured in control fish	18 °C		26 °C/27 °C	Analysis of variance (ANOVA)			
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Experiment	Temperature	Exp. X temperature
Glucose (mg/dL)	31.2 ± 11.1	89.8 ± 11.1	43.1 ± 11.6	71.5 ± 20.7	**F(1,63) = 9.4 P = 0.003**	F(1,63) = 0.05 P = 0.82	F(1,63) = 1.13 P = 0.29
Cortisol (μg/mL)	38.6 ± 7.2	13.2 ± 7.8	35.7 ± 7.8	22.6 ± 13.6	**F(1,59) = 4.1 P = 0.05**	F(1,59) = 0.12 P = 0.74	F(1,59) = 0.42 P = 0.52
Vitellogenin (ng/mL)	157 ± 6.5[a]	63 ± 4.5[b]	118 ± 20[a]	225[b]	F(1,59) = 0.04 P = 0.84	**F(1,59) = 10.1 P = 0.002**	**F(1,59) = 24.3 P < 0.0001**
Spermatocytes (%)	18.3 ± 1.0 ns	16.5 ± 0.9 ns	5.6 ± 0.6[a]	23.0 ± 1.6[b]	**F(1,3) = 75.8 P = 0.003**	**F(1,3) = 28.5 P = 0.01**	**F(1,3) = 103.2 P = 0.002**
Spermatids (%)	3.0 ± 0.3	5.6 ± 0.4	1.2 ± 0.1	3.4 ± 0.4	**F(1,3) = 47.1 P = 0.006**	**F(1,3) = 35.1 P = 0.01**	F(1,3) = 2.6 P = 0.21

treatment combinations, for both Exp. 1 & 2, HCT tended to be higher with ff, high E1, 18 treatments.

At first glance, these results appear to be unexpected, as often elevated hematocrit occurs in response to lower dissolved oxygen associated with higher water temperatures (Gallaugher et al., 1995), but due to tank aeration, DO concentrations should have been similar to those at 18 °C. This is supported by data from previous laboratory experiments (data not shown), where dissolved oxygen in tanks with water at 18.9 °C was 8 ± 1.5 mg/L, and 7 ± 0.2 mg/L at 24.4 °C, both greater than the >5 mg/L established for FHM culture (U.S. Environmental Protection Agency, 2002). Literature on the effect of estrogens on hematocrit in fish is inconsistent, and previous results from this laboratory using fathead minnows fail to clarify the picture, as extremely high E1 (390 ng/L) actually decreased hematocrit (Feifarek et al., accepted). One study has reported a non-significant increase in hematocrit (20%) in female red grouper receiving intra-peritoneal injections of 17β-estradiol, every other d for 9 d (Ng et al., 1984).

3.3.2.2. Blood glucose. While lower blood glucose concentrations would be expected with food restriction, a significant treatment effect was only seen in Exp. 2 ($P = 0.02$). In Exp. 1, food restriction resulted in a 20% reduction of mean blood glucose compared to full fed (though not significant, mean not shown, Table 1), while in Exp. 2 mean glucose of fish on food restriction was reduced by 29% (Table 2).

3.3.2.3. Cortisol. Cortisol values were highly variable, with no significant differences due to treatment (SI Table 7), with means ranging from 34 to 52 pg/mL in Exp. 1 (Table 1), and 13 to 27 pg/mL in Exp. 2 (Table 2).

3.4. Difference between control treatments in experiments 1 and 2

Replication of experiments in ecotoxicological publications from three journals (2013 publications) was recently evaluated and found to be greatly lacking (Harris and Sumpter, 2015). Of 179 papers, experiments had been performed once in 53%, 71% or 85% of the papers for the three journals. Duplication of experiments here, resulted in the observation of differential response of several parameters in Exp. 1 vs. 2 for control full fed fish, either at 18 °C or ~26 °C. Analysis of all full fed control fish from each experiment were compared and also compared at either high or low temperatures (Table 5). While in theory all experimental conditions were consistent from Exp. 1 to Exp. 2, blood glucose was lower in Exp. 1 (mean of temperatures, 37 vs 81 mg/dL, $P = 0.003$). In contrast, plasma cortisol concentration was higher in Exp. 1 (mean of temperatures, 37 vs 18 pg/mL, $P = 0.05$). These results could have been the result of differential handling at time of sacrifice or may reflect differential set points. In either case, increases in cortisol can result in temporary increases in blood glucose (Pottinger and Carrick, 1999; Jentoft et al., 2005).

Control values for mean plasma VTG concentrations also varied by experiment, with differential response to temperature ($p <$ 0.0001). At 18 °C, VTG was higher in Exp. 1 control fish than Exp. 2 (157 vs 63 μg/mL, Table 5). At 26 °C the results were reversed: VTG was lower in Exp. 1 control fish than Exp. 2 (118 vs 225 ng/mL Table 5. For spermatocytes no differences were found at 18 °C between Exp. 1 and 2, but at ~26 °C controls were higher in Exp. 2 ($P = 0.004$). An effect of experiment and temperature was also seen for spermatids (Table 5). The mean percent spermatids for control fish in Exp. 1 was lower than in Exp. 2 (1.9 vs. 4.3 $P = 0.006$, data not shown). Mean at 18 °C was higher than at ~26 °C (4.1 vs. 2.0, data now shown). The decrease in more mature stages of sperm at higher temperatures would agree with Brungs's (1971) reported decline in spawning as temperatures increased above 23.5 °C, and no spawning observed at 32.5 °C.

The differences in mean values for control fish may indicate the presence of some inherent seasonality that cannot be blocked even by removing environmental cues such as photoperiod or water temperature. Though not statistically tested, in three other sets of replicate experiments performed in this laboratory, back to back in late fall to early winter, the mean VTG value for control fish was less than the mean for control fish in the earlier experiment (both male and female fish in Dammann et al., 2011; male fish from separate replicated experiments in companion manuscript). As the calendar moves further from the natural spring/summer breeding time, FHM appear to enter a recalcitrant state relative to reproductive status. The differential distribution of mature spermatozoa would appear to support this hypothesis, as sperm maturation decreased in later months. Smith (1978) documented status of sperm maturation of FHM in the wild, with similar findings of decreased mature sperm (spermatozoa) with the onset of fall (shortened photoperiod and reduced water temperatures). In addition to personal communications reporting the same laboratory "seasonal" quiescence for fish, similar findings were observed in some species of quail (personal communication, Mary Ann Ottinger, 2017). Lack of physical cues may also be the cause of such quiescence. While laboratories adjust photoperiod to mimic breeding seasons, they lack the incremental changes in seasonal photoperiods, as well as the diurnal and seasonal gradients in water temperature found in nature.

4. Conclusions

While the intent of this study was to evaluate the how abiotic and biotic environmental factors such as water temperature and food restriction affect endocrine disruption caused by exposure to E1, several unexpected findings were uncovered. Most unexpected was that our "high" temperature (26 °C, which is in line with 25 ± 1 °C recommended for both general laboratory toxicity and reproductive toxicity studies of FHM, Denny, 1987; U.S. Environmental Protection Agency, 2002) altered both morphological parameters and sperm differentiation independent of E1 exposure. High temperature resulted in a decrease in three of eight or eight of eight morphological endpoints (Exp.1 and 2, respectively) compared to fish held at 18 °C. Of these endpoints, only liver weight and HSI were affected by E1 in experiment 2, with the

highest means found in high E1 treatment at 18 °C. Similarly, the highest mean hematocrit was for full fed fish at 18 °C exposed to high E1. Three out of four measurements of sperm differentiation were altered by high temperatures, though E1 also impacted differentiation either alone or in combination with temperature. These findings corroborate previous observations from whole effluent exposures (Vajda et al., 2008) reporting that estrogens may alter the status of sperm maturation of fathead minnows. These findings are particularly pertinent in light of recent reports that emphasize the relevance and in vivo potency of E1 as an environmental estrogen (Ankley et al., 2017). Given the observed interactions of temperature and E1 in changing biomarker expression, it is critical that E1 is evaluated in more detail over the range of breeding temperatures for this species. As fish in natural environments are exposed to a multitude of stressors, including diurnal and seasonal alterations in temperature, feed limitation and exposure to other EDs, an assumption can be made that our findings are conservative and may not fully capture the environmental cost of EDs on fish populations. Plasma VTG was elevated only with high E1 exposure in both experiments. Finally, a "pseudo-seasonality" effect appeared in control values for full fed fish between Exps. 1 and 2 for blood glucose, cortisol, VTG, and status of sperm maturation. This study indicates the need to re-evaluate temperatures at which FHM studies are conducted, and indicates that higher, yet common environmental temperatures might have a greater population impact than the presence of high concentrations of natural estrogens.

Disclosure

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.08.021.

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